Supporting Information

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SI Methods

Cell Culture. Human embryonic stem cells (hESCs; lines H9.2 passages 32–61, I3 passages 55–82, and I6 passages 42–55) were maintained on irradiated mouse embryonic fibroblasts (MEFs) at 5% CO_2 in medium containing Knockout-DMEM (KO-DMEM, Invitrogen), 20% serum replacement, 1% non-essential amino acids, 1 mM L-glutamine, 0.1 mM ß-mercaptoethanol and 4 ng/mL FGF2 (all Invitrogen). Cultures were manually passaged at a 1:3–1:6 split ratio every 4–7 d.

Neural differentiation was performed as previously described (1, 2) with slight modifications. Briefly, 4-day-old embryoid bodies were transferred to polyornithine-coated tissue culture dishes and propagated in ITSFn medium (DMEM/F12; Invitrogen), 25 μg/mL insulin, 100 μg/mL transferrin, 5 ng/mL sodium-selenite (all Sigma–Aldrich), 2.5 μg/mL fibronectin (MP Bio-medicals). Within 10 days, neural tube-like structures developed in the embryoid body outgrowth. First neural differentiation appears with small rosettes showing a columnar shape but without 3-dimensional growth. These neural islands eventually become further organized and increase in size forming neural tube like structures with a central lumen and 3-dimensional growth. These structures were mechanically isolated by separating the island from the surrounding cells with a scalpel or needle. Mechanical selection minimizes contamination with non-neural cells, which can be also found in the vicinity of the neural islands. Isolated clusters where further propagated as free-floating neurospheres in DMEM/F12 (Invitrogen) supplemented with N2 supplement (1:100; Invitrogen) containing 10 ng/mL FGF2 (R&D Systems) for 1 to 7 days. Spheres were triturated into single cells by incubating the spheres with trypsin/EDTA for 10 min followed by gentle dissociation with a 1.000-μL pipette tip. Cells were plated on polyornithine/laminin (both Sigma–Aldrich) precoated plastic dishes. Media was changed to neural stem cell medium (NSCM) containing DMEM/F12, N2 supplement (1:100; both Invitrogen), 20 μg/mL additional insulin (Sigma–Aldrich), 1.6 g/L glucose, 10 ng/mL FGF2 (R&D Systems), 10 ng/mL EGF (R&D Systems), and 1 μL/mL B27 supplement (Invitrogen). High cell densities were essential during initial plating and in the subsequent first passages. Media was changed daily during the first 7 days and every other day thereafter; growth factors were always provided on a daily basis. Passaging was performed only at very high cell density, typically 1 day after the cells had reached full confluence. During the first 5 passages, cells were split at a 1:2 ratio using trypsin/EDTA (1:2–1:3 ratio at later passages). Trypsin was inhibited by trypsin-inhibitor (Invitrogen) and cells were centrifuged at 300 × *g* for 5 min at 4 °C in a Megafuge 1.0R (Heraeus). Replating densities were kept >30%.

Terminal differentiation was performed in DMEM/12 (N2 supplement; 1:50) and Neurobasal (B27 supplement; 1:50) mixed at a 1:1 ratio. cAMP (300 ng/mL, Sigma–Aldrich) was added to the media (referred to as differentiation media).

For induction of ventral midbrain phenotypes, cultures were incubated with DMEM/F12 (N2 supplement; 1:100) with addition of 200 ng/mL SHH, 100 ng/mL FGF8b (both R&D Systems), and 160 μM ascorbic acid (Sigma–Aldrich) for at least 8 days. Differentiation was performed for 14 days in differentiation media described above. BDNF (20 ng/mL), 10 ng/mL GDNF (both R&D Systems), 160 μM ascorbic acid, and 0.5 mM dibutyryl-cAMP (both Sigma–Aldrich) were added in these experiments. For induction of more posterior phenotypes, 1 μM retinoic acid (Sigma–Aldrich) was added to NSCM for 6 days in the presence of additional B27 supplement (1:50). Ventral spinal cord phenotypes (including motoneurons) were generated by adding 1 μg/mL SHH from day 5. From day 7, media was changed to NSCM (without FGF2 and EGF) but with B27 (1:50), 1 μg/mL SHH and 0.01 μM RA for another 6 days. SHH was reduced to 50 ng/mL for another 7 days, and cells were terminally differentiated in the presence of 20 ng/mL BDNF and 20 ng/mL GDNF in differentiation media.

Clonal Analysis. We used an automated system (CytoClone; Evotech Technologies), which permits the gentle deposition of single cells in individual wells of 96-well plates under real-time documentation. Wild-type cells and lt-hESNSCs harboring an EGFP expression construct were triturated to a single cell suspension by treatment with trypsin/EDTA (5 min at 37 °C). Cells were suspended at 5 × 10⁵ cells per mL in Cytocon Buffer II (Evotec Technologies), and 3–5 μL of the cell suspension were loaded into the CytoClone (Evotec Technologies) as described in ref. 6. Target cells were caged in the dielectric field cage of the CytoClone Sorter Chip (Evotec Technologies), followed by phase contrast image analysis to obtain cell characterization and confirmation of single cell status. Fluorescence imaging was used to confirm the status of EGFP expression. The cells were imaged using a 40× lens. The average diameter of selected cells was 12 μm. Cells were deposited on irradiated mouse astrocyte feeder cells (15 gray) generated from murine ES cells as described before (3). Cultures were incubated with NSCM for 14 days and subsequently differentiated for 4–12 weeks. Clones were detected by virtue of their EGFP expression and/or an antibody to human nuclear protein.

Immunocytochemistry. Cells were fixed in 4% neutral-buffered paraformaldehyde (PFA) for 20 min or acetone/ethanol (75:25) for 5 min at room temperature. For detection of gamma-aminobutyric acid (GABA), 0.05% glutaraldehyde (Sigma–Aldrich) was included in the fixative. Cells were permeabilized with 0.1% Triton X-100 (Sigma–Aldrich) in PBS for 20 min. Blocking was performed with 10% FCS (Invitrogen) in PBS for 1 h. Samples were incubated with primary antibodies at room temperature for 3–4 h, washed twice, incubated with secondary antibody for 45 min, counterstained with DAPI, and mounted with Vectashild mounting solution (Vector Laboratories). Tissues were prepared from animals perfused with 4% PFA. Tissues were postfixed in PFA for 6 h, then cryoprotected in 30% sucrose and sectioned at 40 μm by using a cryostat. Sections were permeabilized/blocked with 0.1% Triton X-100 + 10% FCS for 1 h at 25 °C. Incubation with primary antibody was for 16 h at room temperature. Incubation with fluorescently tagged secondary antibodies was for 2 h at 25 °C. All antibodies, sources, and dilutions are listed in Tables S1 and S3.

Statistics. For determination of phenotypes after differentiation in vitro duplicate samples of each passage and cell line were differentiated and experiments were performed at least 3 times. Numbers were determined by counting at least 1,000 cells per sample in randomly picked fields. For determination of phenotypes in vivo at least 150 cells per animal (*n* = 3 per time point) were counted for every marker. Values represent means in % ± SEM.

Karyotyping and Fluorescent in Situ Hybridization (FISH). Colcemid (Invitrogen) was added to approximately 50% confluent cultures of lt-hESNSCs at a concentration of 0.2 μg/mL for 6 h. Cells were then rinsed twice with PBS, trypsinized, and centrifuged in DMEM. KCl (0.075 M) was added to the pellet, and the cells

were incubated for 10 min at 37 °C, centrifuged and fixed with 3:1 methanol:acetic acid for 10 min, then centrifuged again and suspended in this fixative. Metaphase spreading was performed as described in ref. 4. Cytogenetic analysis was conducted on metaphase cells in passages 20, 40, 60, and 80 using G-banding on a minimum of 30 cells. A $100\times$ oil-immersion objective was used to visualize the spreads, which were further analyzed by Cytovision Software (Applied Imaging). Since long-term expanded hESCs are known to be particularly prone to trisomies 12 and 17 (4), an additional 200 interphases were subjected to interphase fluorescence in situ hybridization (FISH) using chromosome enumeration probes (CEP) for both chromosomes (Vysis, alpha satellite DNA, 12p11.1-q11/32-132012 and 17p11.1-q11.1/32-130017).

TRAP Assay. Non-radioactive telomere amplification protocol (TRAP) was performed using an appropriate kit with supplied protocol (Trapeze, Chemicon).

Lentiviral Transduction. lt-hESNSCs were transduced with pLentiPGK-EGFP-SV40-basticidine, a lentiviral expression construct based on the pLenti6/V5 expression system (Invitrogen) where the CMV promoter was replaced by a phosphoglycerate kinase (PGK) promoter element (gift from Harald Neumann) and subsequently selected for blasticidine resistance. Lentivirus production and transduction of the cells were performed as described in ref. 5.

Transplantation. Neonatal SCID-beige mice (Harlan), within 48 h of birth, were cryoanesthetized with ice. Approximately 2 μL of cell suspension containing 50,000 cells per μL was slowly injected bilaterally into the telencephalon using a glass micropipette. The animals were observed on a daily basis to exclude development of obvious neurological deficits. Tissue analysis was performed at 3, 6, 9, 12, 15, 18, and 24 weeks after transplantation.

RT-PCR. Triplicate total mRNA samples were isolated using a mRNA extraction kit (Qiagen), following the supplier's instructions. Between 0.5–1 μg of total RNA were used for reverse transcription with the iScript cDNA synthesis kit (BioRad), following the manufactor's protocol. Reactions were run in at least triplicate using Taq Polymerase (Invitrogen). To compare the expression level of different genes, probes were normalized to GAPDH by performing 15, 20, and 25 cycles. PCR conditions and cycle numbers were established by using commercially available human fetal (single donor, female, 19 weeks of gestation) or adult brain probes (both Stratagene). The selected number of cycles varied from 35 to 40 cycles depending on the particular mRNA abundance with denaturation at 94 °C for 1 min, annealing temperatures at 58 °C to 63 °C for 60 seconds according to the primers, and elongation at 72 °C for 2 min. Omission of transcriptase during RT or cDNA sample during PCR served as negative controls. All reactions were performed on a T3 Thermocylcer (Biometra). Primers used are listed in Tables S2 and S4.

Electrophysiological Recordings of Cultured lt-hESNSCs. Cells grown on 13-mm diameter glass or plastic coverslips were transferred to a chamber that was mounted to an x-y stage and continuously superfused with aCSF at 1–2 mL/min. This aCSF contained the following (in mM): 140 NaCl, 3 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 25 D-glucose, and 10 Hepes/NaOH (pH 7.35, 305–315 mosmol/kg). Recordings were performed at room temperature. Cells were visualized using an upright microscope equipped with near-infrared differential interference contrast (IR-DIC) and $40\times$ water-immersion objective (Zeiss). Whole cell current-clamp and voltage-clamp recording was carried out with an Axopatch-200B amplifier (Axon Instruments) that was interfaced by an A/D-converter (Digidata 1320, Axon Instruments) to a PC running PClamp software (Version 9, Axon Instruments). For most recordings of membrane potential or current, the patch pipette (tip resistance 3–5 MΩ) contained the following (in mM): 120 potassium gluconate (C$_6$H$_{11}$O$_7$K), 20 KCl, 10 NaCl, 10 EGTA, 1 CaCl$_2$, 4 Mg ATP, and 0.4 Na GTP, and 10 Hepes/KOH (pH 7.2, 280–290 mosmol/kg). For some voltage-clamp recording, another pipette filling solution was used (in mM): 110 cesium methanesulfonate (CH$_3$O$_3$SCs), 10 CsCl, 10 NaCl, 10 TEA-Cl, 10 EGTA, 1 CaCl$_2$, 4 Mg ATP, and 0.4 Na GTP (pH 7.2, 280–290 mosmol/kg). For the latter solution, the holding potential was corrected for a 9-mV junction potential.

Electrophysiological Recordings of Transplanted lt-hESNSCs. Animals that had received transplants at P1 were deeply anesthetized with a mixture of ketamin and xylazine, and transcardially perfused with ice-cold solution containing (in mM) 80 NaCl, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 1 CaCl$_2$, 5 MgCl$_2$, 30 NaHCO$_3$, 25 mM D-glucose, and 75 sucrose (gassed with 95% O$_2$/5% CO$_2$, pH 7.4, 300–305 mosmol/kg). The brain was then rapidly removed, trimmed by cutting off cerebellum and hindbrain directly behind the occipital cortex, and glued to the stage of a vibratome (Mikrom) to cut 300 μm thick coronal slices in the same solution at 4 °C. Slices were incubated after cutting in solution containing (in mM) 85 NaCl, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 1 CaCl$_2$, 5 MgCl$_2$, 25 NaHCO$_3$, 25 D-glucose, and 75 sucrose (gassed with 95% O$_2$/5% CO$_2$, pH 7.4, 300–305 mosmol/kg) for 20 min and stored thereafter at room temperature for up to 6 h. For recording one slice at a time was transferred to a chamber and continuously superfused with aCSF at 1–2 mL/min. This aCSF contained the following (in mM): 125 NaCl, 5 KCl, 1.25 NaH$_2$PO$_4$, 2 CaCl$_2$, 1 MgCl$_2$, 25 NaHCO$_3$, and 25 D-glucose (gassed with 95% O$_2$/5% CO$_2$, pH 7.4, 300–305 mosmol/kg). Recordings were performed at 32 °C. Transplanted cells were identified by their EGFP fluorescence and visualized using an upright microscope equipped with near-infrared differential interference contrast (IR-DIC) and $40\times$ water-immersion objective (Zeiss). For recordings of membrane potential or current, the patch pipette [tip resistance 3–5 MΩ) contained the above mentioned, potassium gluconate-based solution. Signals were filtered at 2 kHz and recorded at a rate of 20 kHz. All electrophysiological recordings were analyzed using Clampfit (vers. 9, Axon Instruments) and Microsoft Excel.

Antibodies and PCR Primers Used to Assess the Purity of lt-hESNSCs. lt-hESNSCs derived from ES cell lines H9.2, I3, and I6 were subjected to immunofluorescence and RT-PCR analysis to exclude contamination with pluripotent cells and differentiation in derivatives of other germ layers. No positive signals were detected. For antibodies and primers, see Tables S1 and S2.

1. Li XJ, Zhang SC (2006) In vitro differentiation of neural precursors from human embryonic stem cells. *Methods Mol Biol* 331:169–177.
2. Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA (2001) In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol* 19:1129–1133.
3. Brustle O, et al. (1999) Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science* 285:754–756.
4. Draper JS, et al. (2004) Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nat Biotechnol* 22:53–54.
5. Koch P, Siemen H, Biegler A, Itskovitz-Eldor J, Brustle O (2006). Transduction of human embryonic stem cells by ecotropic retroviral vectors. *Nucleic Acids Res* 34:e120.
6. Koch P, et al. (2005) Automated generation of human stem cell clones by Image-Activated Cell Selection (IACS™), *Nat Methods*, 10.1038/nmeth809.
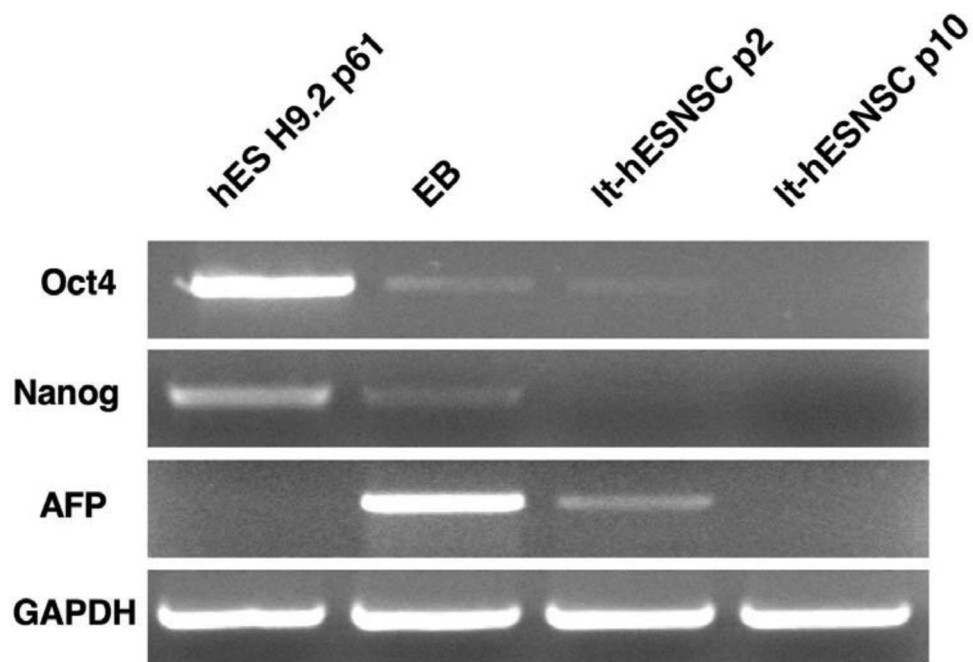
Fig. S1. Loss of pluripotency markers during generation of lt-hESNSCs. RT-PCR analysis revealed that EBs and hESC-derived precursors at passage 2 still contain cells expressing pluripotency markers such as Oct4 and Nanog as well as markers for other germ layers such as alpha-fetoprotein (AFP). These contaminating cells are no longer detectable in passages >10.
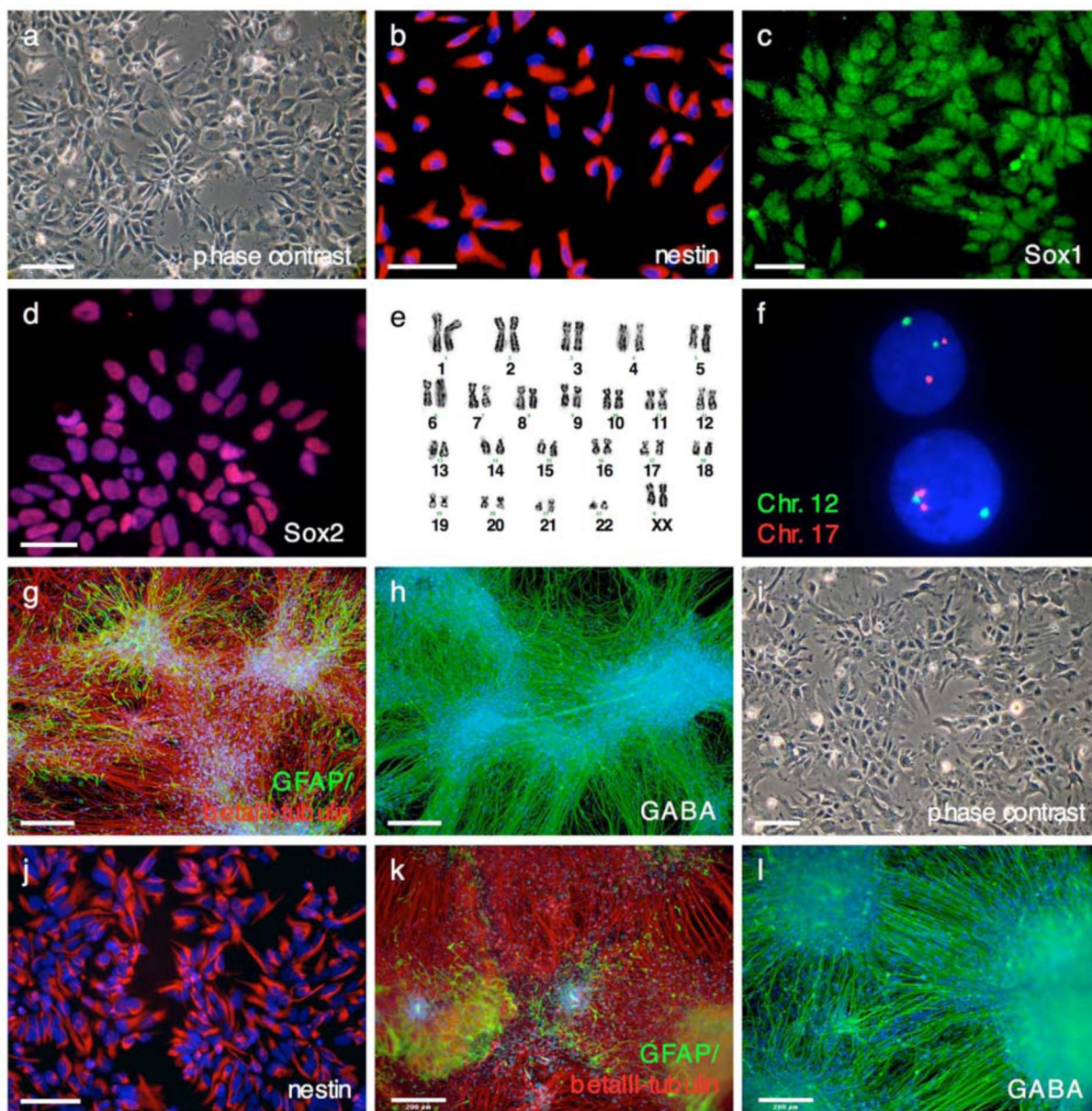
Fig. S2. The generation of lt-hESNSCs is independent of genetic background. Lt-hESNSCs derived from 2 additional parental hES cell lines I3 (*A–H*) and I6 (*I–L*) show comparable morphology (*A* and *I*), karyotypic stability (*E* and *F*) and neural marker expression in the proliferating (*B–D*, *J*) and differentiated state (*G* and *K*). Similar to their counterparts derived from the parental line H9.2, they exhibit a strong propensity for GABAergic differentiation (*H* and *L*). (Scale bars, *A*, *B*, *I*, and *J*: 50 μm; *C* and *D*: 25 μm; *G*, *H*, *K*, and *L*: 200 μm.)

Fig. S3. Lt-hESNSCs exhibit stable proliferation and extensive telomerase activity. (*A*) Growth curves from H9.2 cells at passages (P) 1–10, 31—40, and 51–60 indicate that after an initial consolidation process the cells exhibit a stable proliferation behavior. To illustrate overall growth potential, starting cell numbers of growth curves for P31–40 and P51–60 have been normalized to the end cell numbers of passages 10 and 40, respectively. (*B*) TRAP (telomere repeat amplification protocol) analysis shows that lt-hESNSCs maintain expression of functional telomerase over at least 60 passages. Note that due to excessive telomerase activity, the 36-bp internal control (IC) band is extinguished in non-heat-treated sample lanes because amplification of the TRAP products and the S-IC control band are semicompetitive.

Fig. S4. Lt-hESNSCs display multipotentiality at a clonal level. To investigate the differentiation potential of lt-hESNSCs at a clonal level, we used the image activated cell selection (IACS, Cytoclone) technique [Koch P, *et al.* (2005) Automated generation of human stem cell clones by Image-Activat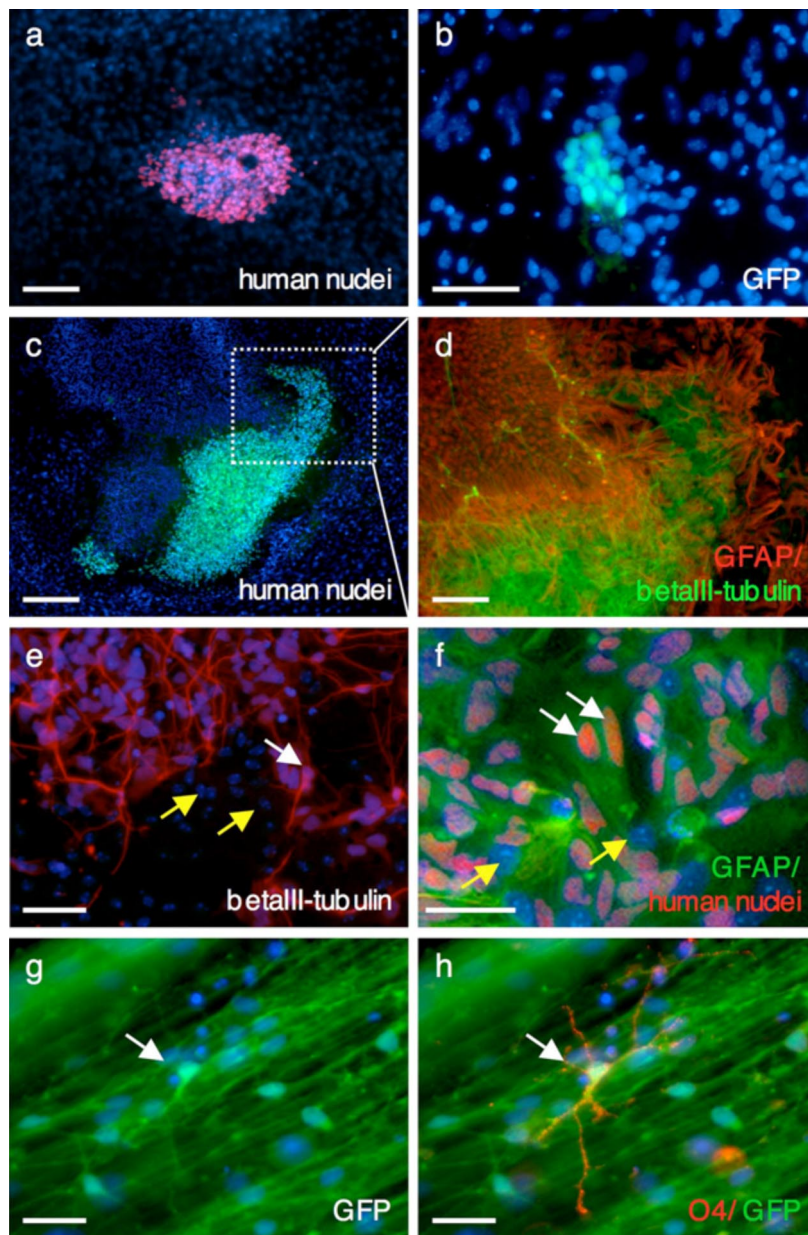ed Cell Selection (IACS™), *Nat Methods*, 10.1038/nmeth809]. Single lt-hESNSCs are caged in a dielectric field, documented with respect to size and fluorescence and individually spotted into single wells of multiwell plates containing a murine astrocyte monolayer. After 14 days of proliferation clonally derived colonies can be detected with an antibody to human nuclei and/or GFP-fluorescence (*A* and *B*). Upon growth factor withdrawal, clones derived from single lt-hESNSCs give rise to neurons and astrocytes (*C*–*F*). Prolonged differentiation times exceeding 8 weeks also lead to the development of oligodendrocytes, thus confirming tripotentiality of single deposited lt-hESNSCs (*G* and *H*). White arrows show human cells expressing beta III-tubulin (*E*), GFAP (*F*), or O4 (*G* and *H*). Yellow arrowheads mark cocultured murine astrocytes (*E* and *F*). (Scale bars, *A*: 100 μm; *B*, and *E*–*H*: 50 μm; *C* and *D*: 200 μm.)
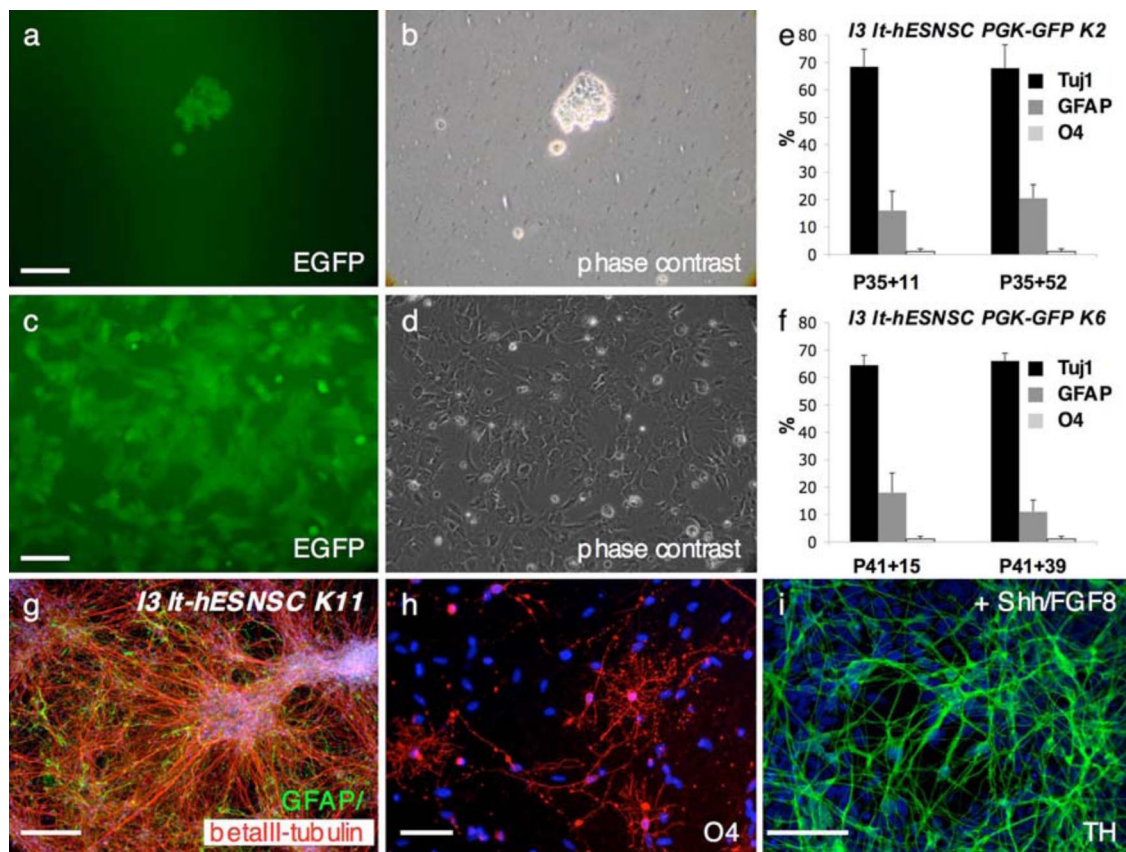
Fig. S5. Clonal lines can be extensively propagated, retain a stable differentiation potential and can be recruited into midbrain fates. To study whether stable neuro- and gliogenesis as well as responsiveness to patterning cues are also preserved at a clonal level, lentivirus-transduced lt-hESNSCs (carrying the PGK-EGFP transgene and a blasticidine resistance gene; passages 31–47) were mixed with wildtype cells at a ratio of 1:100,000. This dilution typically resulted in approximately 1 resistant cell per cm². Two days after transduction wildtype cells were eliminated by treatment with 4 μg/mL blasticidine for another 5 days. (*A* and *B*) PGK-GFP-positive clone 6 days after addition of blasticidine. An average number of 0.27 clones/cm² were obtained. Clones isolated and further propagated for up to 60 passages showed homogeneous morphology and EGFP transgene expression (*C* and *D*). (*E* and *F*): Four weeks after growth factor withdrawal clonal lines differentiated into neurons, astrocytes and oligodendrocytes at levels comparable to bulk preparations (see also Fig. 1*I–O*). This differentiation pattern was independent of the number of passages the individual clones were subjected to. Depicted are percentages of beta-III tubulin(+) neurons, GFAP(+) astrocytes, and O4(+) oligodendrocytes in clonal lines generated from lt-hESNSCs at passage 35 (*E*) or 41 (*F*) and cultured for another 11 and 52 or 15 and 39 passages, respectively. In addition clones were generated by automated single cell spotting as described [Koch P, *et al.* (2005) Automated generation of human stem cell clones by Image-Activated Cell Selection (IACS™), *Nat Methods*, 10.1038/nmeth809] and propagated for up to 45 passages. (*G*) and (*H*) depict multipotent differentiation of a clonal line generated from a single deposited cell (from passage 38 bulk culture) and cultured for another 15 passages before initiation of differentiation by growth factor withdrawal. The cells generate neurons and astrocytes (*G*) and oligodendrocytes (*H*). After exposure to Shh and FGF8 for 10 days this clonal line gave rise to a prominent fraction of TH-positive neurons (*I*). (Scale bars, *A–D*, *H*, and *I*: 50 μm; *G*: 200 μm.)
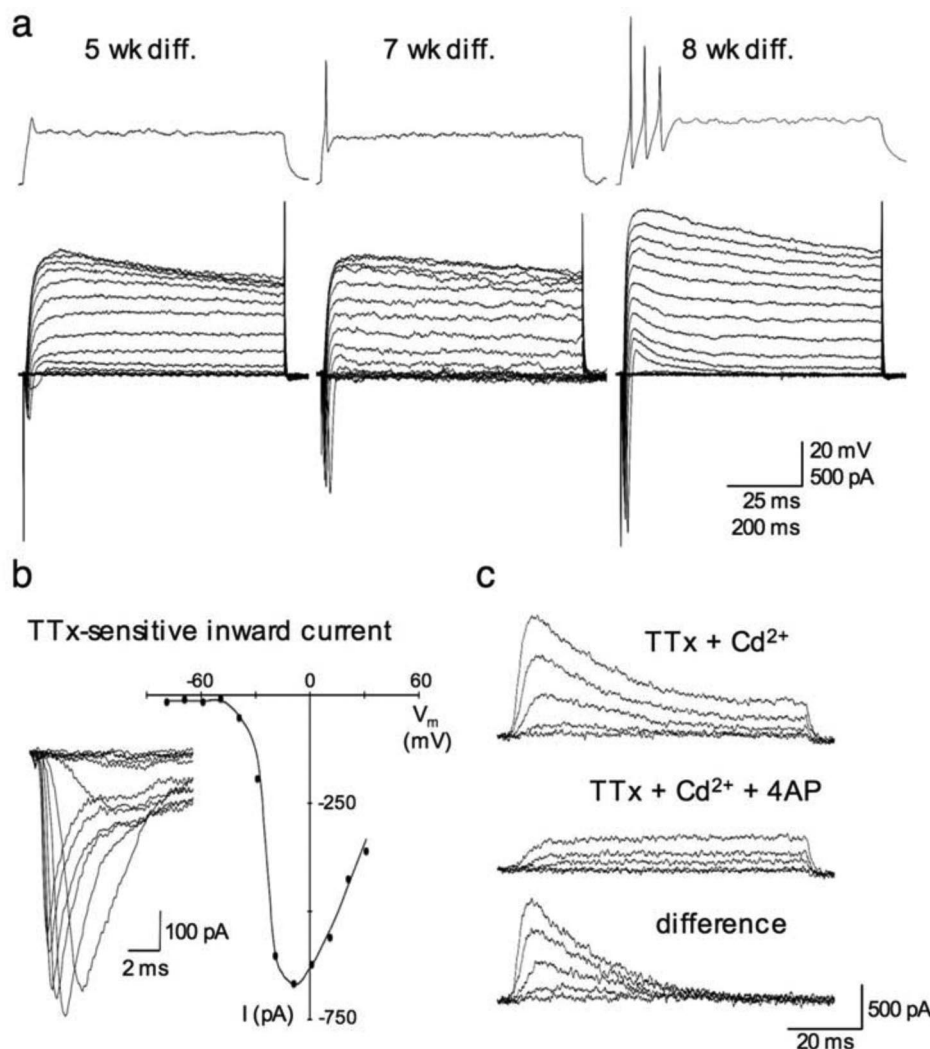
Fig. S6. Functional maturation of lt-hESNSC-derived neurons in vitro. Analysis of whole-cell currents revealed developmental maturation of both voltage-dependent inward and outward currents in cells that were differentiated for 5–8 weeks (*A*). We observed an increase of the inward current amplitude, which was paralleled by the cells' ability to fire an action potential when depolarized in current-clamp experiments. Also the outward current increased in amplitude during differentiation and additionally became more complex by developing an inactivating component. These further changes of active membrane properties facilitated repetitive firing of the neurons in current clamp. The fast and transient inward current could be isolated by use of intracellular Cs^+, extra- and intracellular TEA, and extracellular Cd^{2+}, was blocked by TTx (300 nM), and exhibited voltage dependence typical for the fast Na^+ current (*B*). Voltage-activated outward current did not only increase in its amplitude during the time course of differentiation, but also changed in kinetics. While younger neurons mostly showed a slow onset and almost no inactivation during the voltage step, in most older neurons (11 out of 15) the outward current exhibited a partial inactivation. Application of 4-aminopyridine (4-AP, 5 mM) revealed the presence of a fast activating and inactivating, 4-AP-sensitive, and a slowly activating and persisting, 4-AP-resistant component reminiscent of A-type and delayed rectifier currents, respectively (*C*).
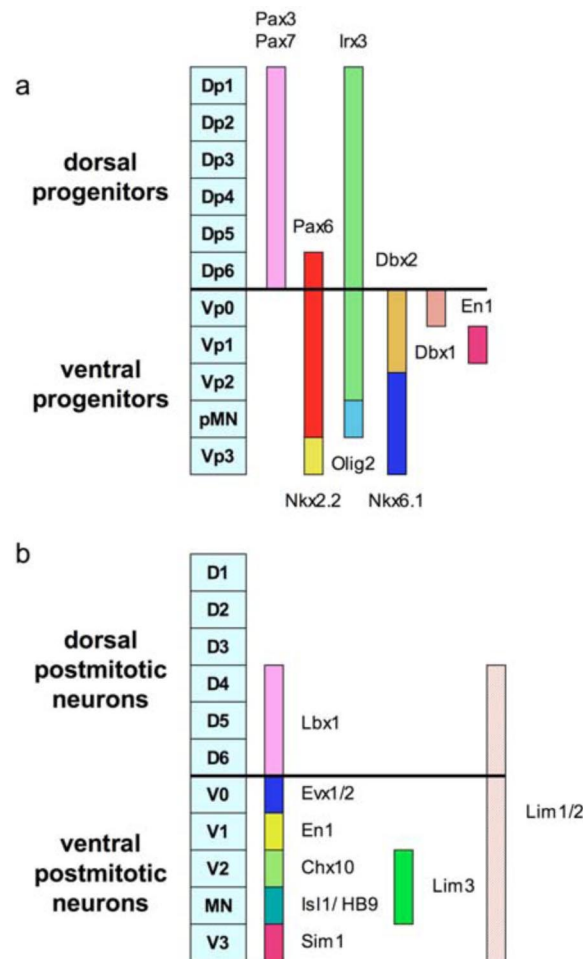
Fig. S7. Schematic drawing outlining transcription factor expression domains across the dorso-ventral axis of the hindbrain. (*A*) Dorsal progenitors (Dp1-Dp6) are characterized by expression of Pax3, Pax7, and Irx3. Within the ventral hindbrain, Vp0 progenitors express Dbx1, Dbx2, Irx3, and Pax6. Vp1 progenitors have a similar homeodomain protein code, but lack Dbx1 and express En1. The boundary between Vp1 and Vp2 is defined by the transcription factors Dbx2 and Nkx6.1. Thus, Vp2 progenitors are characterized by expression of Nkx6.1 but also Irx3 and Pax6. Motoneuron progenitor cells in the pMN domain express Nkx6.1 and Pax6 but lack Irx3. In addition, they express the basic helix–loop–helix (bHLH) transcription factor Olig2. Very ventral interneurons arise from Nkx2.2(+) progenitors, some of which also express Nkx6.1 but lack Pax6. (*B*) Upon differentiation ventral progenitors give rise to the V0–V3 interneurons and pMN-derived motoneurons. Similar to their progenitors, postmitotic neurons can be subdivided by virtue of their transcription factor code. V0 interneurons are characterized by the expression of Evx1/2. V1 interneurons are defined by expression of En1 whereas V2 neurons express Chx10 and GATA2. Lhx3 (Lim3) is expressed by V2 interneurons but also by some motoneurons, which can be further identified by expression of Isl1 and HB9. pV3 progenitors give rise to Sim1-expressing ventral interneurons, some of which also express Isl1. The transcription factor Lhx1/5 (Lim1/2) is mainly expressed in V0–V2 interneurons even though its expression domain extends to more ventral interneuronal and dorsal populations.
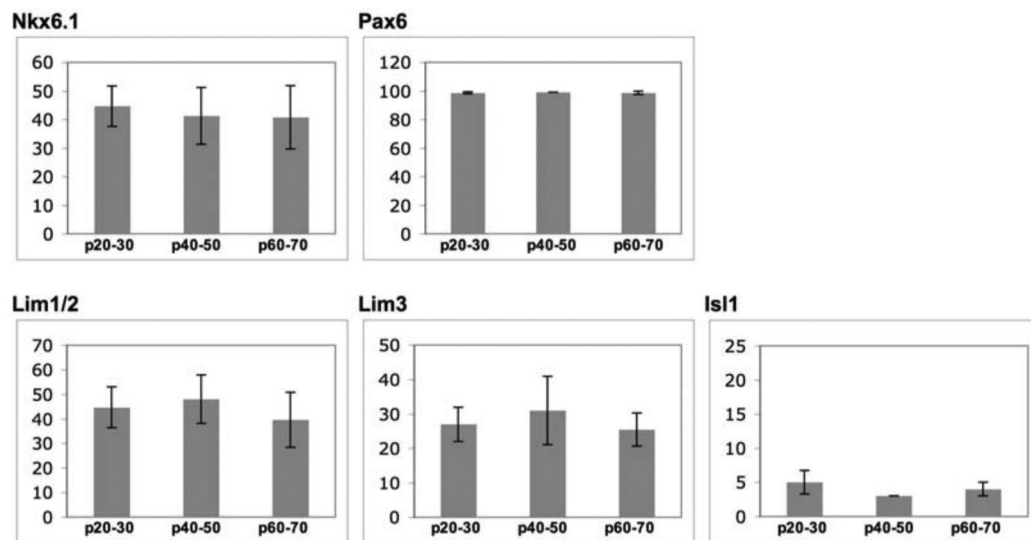
Fig. S8. Regional marker expression remains stable over the passages. Expression of Nkx6.1 and Pax6 (proliferating state) as well as Lhx1/5 (Lim1/2), Lhx3 (Lim3), and Isl1 (differentiated state) shows no significant differences between passages p20–30, p40–50, and p60–70.
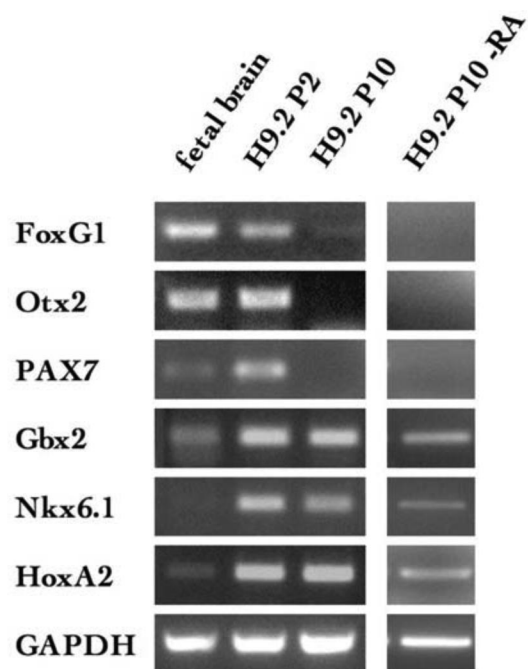
Fig. S9. Derivation of lt-hESNSCs coincides with the acquisition of a regional restriction. Comparative RT-PCR analysis of hESC-derived neural precursors in passages P2 and P10 reveals that freshly isolated neural precursors (P2) still express the anterior markers FoxG1 and Otx2 and the dorsal marker Pax7. Expression of these transcription factors is no longer detectable in established lt-hESNSCs (P10). Comparable results were observed when the cells were cultured in media containing B27 supplement without retinoic acid (-RA).
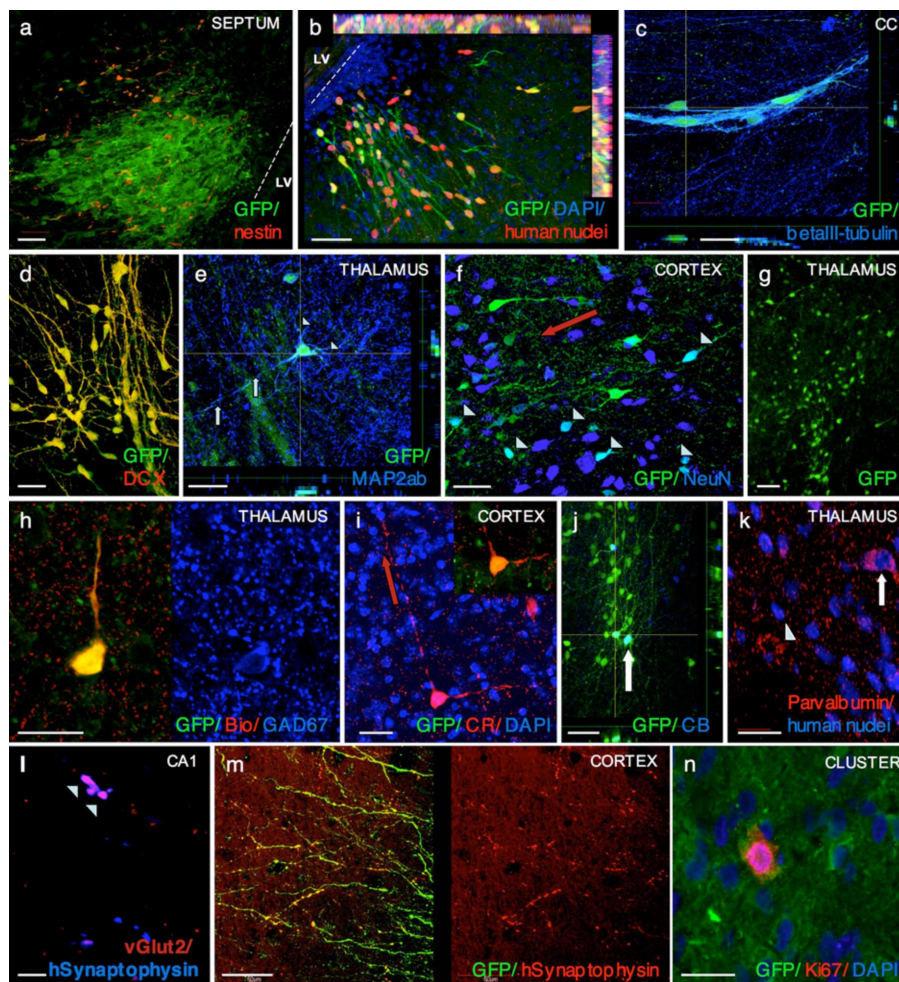
Fig. S10. Incorporation of GFP-labeled lt-hESNSCs in the brain of newborn of SCID-beige mice. Three weeks after transplantation, cells could be detected in many brain regions, including cortex, corpus callosum, thalamus, septum, striatum, hippocampus, and mesencephalon. At this time point, most of the cells were located in clusters adjacent to ventricle walls (*A*). From there, individual cells migrated into the brain tissue (*B*). The majority of grafted cells expressed beta III-tubulin (74.4 ± 6.9%) and DCX (78.6 ± 4.0%), which are markers of early neuronal differentiation (*C* and *D*). Nestin-positive cells were preferentially detectable in periventricular locations (7.0 ± 4.6%) and within clusters of grafted cells (18.2 ± 5.7%) where 3.1 ± 1.5% of the cells stained positive for the proliferation-associated marker Ki67. We could not detect glial differentiation (GFAP) at this time point. 100% of the GFP-expressing cells coexpressed the human nuclei protein at any point of time (*B*). At 4 months after transplantation most neurons exhibited complex neuronal morphologies and expression of the mature neuronal markers MAP2ab (*E*) or NeuN (*F*). By 18 weeks after transplantation NeuN-expression was detected in 62.6 ± 7.6% of the cells. The donor neuron depicted in (*E*) is located in the host thalamus and displays a pyramidal-like phenotype with dendritic processes (arrowheads) and a delicate axon (arrows). The NeuN-positive neurons depicted in (*F*) (arrowheads) have engrafted in the cortex of a 4-month-old recipient. Note the perpendicular orientation to the pial surface (indicated by red arrow) and the small size of the donor neurons. (*G*) Low magnification overview depicting the density of donor cells integrated in the host thalamus. In analogy to the in vitro differentiation data engrafted cells acquired predominantly GABAergic phenotypes. Sixteen weeks after transplantation, cytoplasmic expression of GAD was detected in 51.4 ± 8.7% of the cells positive for human nuclei or GFP (*H*). Further characterization revealed immunoreactivity for calbindin in 13% of GFP-positive cells (*J*) and expression of calretinin (*I*) and parvalbumin (*K*) in 28% and 11% of human nuclei-positive cells, respectively. All 3 calcium-binding proteins are known to be also expressed in subclasses of inhibitory neurons in the hindbrain area (7). The punctuate staining pattern obtained by anti-vGLUT2 immunolabeling precluded a detailed quantification of the glutamatergic lineage. However, colocalization of a subset of human synaptophysin-positive punctae with the vGLUT2 signal revealed the presence of glutamatergic differentiation in vivo (*L*). A survey of markers for other neuronal lineages (TH, serotonin, ChAT) revealed no positively labeled donor cells. A prominent feature in all transplant recipients was the widespread innervation of the host tissue by donor-derived axons. Human axons originating both from periventricular clusters and single integrated cells projected preferentially into white matter tracts as the corpus callosum (*C*), the internal capsule and further caudal into the cerebral peduncles, thereby easily spanning distances of several millimeters. Emanating from these white matter tracts, donor-derived axons frequently branched out into neighboring gray matter where immunolabeling with a human-specific antibody to synaptophysin yielded a punctuate staining pattern characteristic of presynaptic markers (*M*). Glial differentiation in vivo was rare. Less than 1% of donor cells were positive for GFAP or S100β. Twelve to 16 weeks after transplantation, <0.3% of labeled donor cells were found to express the proliferation-associated marker Ki67 (*N*). Abbrevations, LV: lateral ventricle; CC: Corpus callosum; DCX: doublecortin; Bio: biocytin; CR: Calretinin; and CB: Calbindin. Red arrows in (*F*) and (*I*) point to the pial surface. (Scale bars, *A*, *C*–*F*, *H*, *I*, *K*, and *N*: 30 μm; *B*, *G*, *J*, and *M*: 50 μm; *L*: 10 μm.)

7. Ren K, Ruda MA (1994) A comparative study of the calcium-binding proteins calbindin-D28K, calretinin, calmodulin and parvalbumin in the rat spinal cord. *Brain Res Brain Res Rev* 19:163–179.

Fig. S11. Representative traces from electrophysiological analysis of a lt-hESNSC-derived neuron 8 weeks after transplantation into the brain of 1-day-old SCID/beige mice. The neuron exhibited large voltage-dependent inward and complex outward currents and was able to fire repetitive action potentials (*Left*). However, no spontaneous synaptic input was detectable at this time point (*Right*).

Table S1. Antibodies for analysis of pluripotency and germ layer differentiation

Antibody	Source	Dilution
Tra-1–60	Chemicon	1:500
Tra-1–81	Chemicon	1:500
Oct4	Santa Cruz SC-5279	1:500
Alpha-fetoprotein	DAKOCytomation	1:200
Pan-cytoceratin (LU-5)	DAKOCytomation	1:1,000
Epithelial membrane antigen	DAKOCytomation	1:100
Desmin	DAKOCytomation	1:300
Smooth muscle actin	DAKOCytomation	1:800

Table S2. Primers for analysis of pluripotency and germ layer differentiation

Antibody	Primer (5′ to 3′)	
	Forward	Reverse
Oct4	cgaccatctgccgctttgag	cccctgtcccccattccta
Nanog	gcttgccttgctttgaagca	ttcttgactgggaccttgtc
AFP	agaacctgtcacaagctgtg	gacagcaagctgaggatgtc
Brachyury T	caaccaccgctggaagtac	ccgctatgaactgggtctc

Table S3. Antibodies used in neural differentiation studies. The following antibodies were used to study the neural differentiation of lt-hESNSC

Antibodies	Source	Dilution
Ki67/MIB1	DAKO/ M7240	1:250
hNestin	R&D Systems/ MAB1259	1:600
Sox1	Chemicon	1:1,000
Sox2	R&D Systems	1:500
Beta III-tubulin	BAbCo/ MM5435P	1:600
Beta III-tubulin	Covance/ Tuj1	1:2000
MAP2ab	Chemicon/ MAB378	1:500
NeuN	Chemicon/ MAB377	1:100
hSynaptophysin	Chemicon/ MAB332	1:1,000
GFAP	ICN Biomedicals	1:200
GFAP	DAKO/ ZO334	1:1,000
S100beta	Sigma/ S3532	1:2,000
S100beta	SWANT	1:2,000
O4	R&D Systems/ MAB1326	1:200
Human nuclei	Chemicon/ MAB1281	1:200
Serotonin	Sigma/ S5545	1:800
TH	Sigma/ T1299	1:800
DAT	Chemicon/ MAB369	1:500
ChAT	Chemicon/ AB144P	1:100
Calretinin	Swant	1:1,000
Calbindin	Sigma/ C9848	1:1,000
Parvalbumin	Swant/ PV-28	1: 5,000
GABA	Sigma/ A2052	1:1,000
vGlut2	Abcam ab18105	1:500
vGlut1	Chemicon/ AB5905	1:2,000
Gad67	Chemicon/ MAB5406	1:200
Gad65/67	Chemicon/ AB1511	1:500
GFP	Abcam/ ab296	1:2,000
DCX	Santa Cruz/ sc8067	1:200
BF1	gift from Lorenz Studer	1:2,000
Pax6	Developmental Studies Hybridoma Bank	1:500
Nkx2.2	Developmental Studies Hybridoma Bank	1:500
Nkx6.1	Developmental Studies Hybridoma Bank	1:500
HB9	Developmental Studies Hybridoma Bank	1:200
Isl1	Developmental Studies Hybridoma Bank	1:300
En1	Developmental Studies Hybridoma Bank	1:200
Lim1/2	Developmental Studies Hybridoma Bank	1:300
Lim3	Developmental Studies Hybridoma Bank	1:300
Pax7	Developmental Studies Hybridoma Bank	1:300
Pax3	Developmental Studies Hybridoma Bank	1:300
HoxB4	Developmental Studies Hybridoma Bank	1:25
PLZF	Calbiochem	1:50
Dach1	ProteinTech	1:100
ZO1	Zymed	1:100
Cy3-goat-anti-mouse	Jackson/Dianova 115–165–044	1:500
FITC-goat-anti-mouse	Jackson/Dianova 715–095–150	1:500
Cy5-mouse	abcam ab6563	1:500
Cy3-goat-anti-rabbit	Jackson/Dianova 111–165–003	1:500
FITC-goat-anti-rabbit	Jackson Immuno Research	1:250
CY5-goat-anti-rabbit	Jackson/Dianova 111–176–003	1:500
Cy3-goat-anti-rat	Jackson/Dianova 112–165–003	1:500
CY5-goat-anti-rat	Jackson/Dianova 112–175–167	1:500
Cy3-donkey-anti-goat	Jackson/Dianova 705–165–147	1:500
Cy3-goat-anti-guinea pig	Chemicon	1:500

Table S4. PCR primers used in neural differentiation studies. The following primer pairs were used to study the neural differentiation of lt-hESNSC

| | Primers (5′ to 3′) | |
	Forward	Reverse
GAPDH	acgacccttcattgacctcaact	atatttctcgtggttcacacccat
Sox1	caatgcggggaggagaagtc	ctctggaccaaactgtggcg
TERT	tggctgcgtggtgaacttg	gcggttgaaggtgagactgg
FoxG1	ccctcccatttctgtacgttt	ctggcggctcttagagat
Emx1	agacgcaggtgaaggtgtgg	caggcaggcaggctctcc
Emx2	cacagaaacggacaacatgg	ctttagacgagggtcgcttg
Otx2	tgcaggggttcttctgtgat	agggtcagagcaattgacca
Dlx1	caaccagcaaatgtctccttctc	cgcacttcaccgccttcc
Dlx2	ctccctcagctctctcctca	tgtgtccaagtccaggctaa
Gsh2	ctcgctcatcatcaaggaca	agtgcaggtgcgaagtgac
Nkx2.1	cgcatccaatctcaaggaat	tgtgcccagagtgaagtttg
En1	gactcgcagcagcctctc	gcctggaactccgccttg
Gbx2	ctcgctgctcgccttctc	gccagtcagattgtcatccg
Krox20	ttgaccagatgaacggagtg	cagagacgggagcaaagc
Nkx6.1	acacgagacccacttttttccg	tgctggacttgtgcttcttcaac
HoxB2	tttagccgttcgcttagagg	cggatagctggagacaggag
HoxA2	ttcagcaaaatgccctctct	taggccagctccacagttct
HoxA1	gggtgtcctactcccactca	ggaccatgggagatgagaga
HoxB1	tcagaaggagacggaggcta	gtggggggtgttaggttctga
HoxB4	acacccgctaacaaatgagg	gcacgaaagatgagggagag
HoxB6	gaactgaggagcggactcac	ctgggatcagggagtcttca
HoxC5	ccccacagttgctctatgct	gcctctaggaccacttgctg
HoxA5	ccggagaatgaagtggaaaa	acgagaacagggcttcttca
Pax3	gaacacgttcgacaaaagca	gcacacaagcaaatggaatg
Pax7	aagattctttgccgctacca	cacagtgcttcggtcacagt
Irx3	acgaggagggaaacgcttat	cgccgtctaagttctccaaa
Pax6	aataacctgcctatgcaaccc	aacttgaactggaactgacacac
Nkx2.2	tgcctctccttctgaaccttgg	gcgaaatctgccaccagttg
Mash1	gtcctgtcgcccaccatctc	ccctcccaacgccactgac
Olig2	cagaagcgctgatggtcata	tcggcagttttgggttattc
Lbx1	gcgacggtatgaccatcttt	cgattctggaaccaggtgat
Lim1(Lhx1)	atcctggaccgctttctctt	gtaccgaaacaccggaagaa
Evx1	cttctccctcttgcaacca	ggcttcggacaaatttgaga
Lim3(Lhx3)	gcaggacactgaggacagaa	accctgggatctggaaactc
Chx10	cccatcagtggagtccagat	tgtgaggcataggacatgga
Isl1	aaacaggagctccagcaaaa	agctacaggacaggccaaga
Sim1	ttgccaacacttcaccatgt	tggtctcctgctgtctgatg
Notch1	actgtgaggacctggtggac	ttgtaggtgttggggaggtc
HES5	gcccgggggttctatgatatt,	gagttcggccttcacaaaag
HEY1	cgaggtggagaaggagagtg	ctgggtaccagccttctcag
Pou3F3	gttctgcgagaccaccatct	cgatagaggtccgcttcttg
RFX4	tctgagacggcaaacatcac	gactcgatgggagactgctc
GPM6A	tgagatggcaagaactgctg	ccaggccaacatgaaaagat
ASCL1	cggccaacaagaagatgagt	tggagtagttgggggagatg
PLZF	ctatgggcgagaggagagtg	tcaatacagcgtcagccttg
DACH1	gtggaaaacacccctcagaa	cttgttccacattgcacacc
MMRN1	cagggagcatcactcagaca	ttgaggccatcttccatttc
PLAGL1	gcctcagtcacctcaaaagc	cttaacctgtggggcaaaga
NR2F1	acaggaactgtcccatcgac	gatgtagccggacaggtagc
DMTR3	ctaccccatctcgtcttcca	actggcttctcgccaaagta
LMO3	gggctccaccctgtacacta	tagtccgtctggcaaaggat
PMP2	caagctaggccaggaatttg	ccacgcccttcattttacat
AQP4	ggaatttctggccatgctta	agacttggcgatgctgatct
SPARCL1	caactgctgaaacggtagca	gaactcttgccctgttctgc
HOP	gcattgacagcttcactcca	ggaaatgctagccacaccat
S100BETA	aaagagcaggaggttgtgga	aggaaaggtttggctgcttt
FAM70A	ccaggacccagaatgtgact	acataatggcaccggttagc
EVI1	cacattcgctctcagcatgt	atttgggttctgcaatcagc
ZNF312	gccttccaccaggtctacaa	ggtacagggagggaaggaag
LIX1	atgagtcactgccagctcct	gtggaggctactgcttcctg
RSPO3	ggcatgaagcagattggagt	ggcaattgtcaaggcacttt
Lmx1a	acatgagcagggctgagact	tggtgtctccttcagcacag
Lmx1b	acgaggagtgttttgcagtgcg	ccctccttgagcacgaattcg
Nurr1	ttctcctttaagcaatcgccc	aagcctttgcagccctcacag
TH	gagtacaccgccgaggagattg	gcggatatactgggtgcactgg
Pax2	caggcatcagagcacatc	gtcacgaccagtcacaac
AADC	acaagtttgtcctgcgcttt	ccacagacagctgagttcca
Ptx3	gtgggtggagaggagaacaa	ttcctccctcaggaaacaatg